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Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis

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Plant growth is greatly affected by drought and low temperature. Expression of a number of genes is induced by both drought and low temperature, although these stresses are quite different. Previous experiments have established that a *cis*-acting element named DRE (for dehydration-responsive element) plays an important role in both dehydration- and low-temperature-induced gene expression in Arabidopsis. Two cDNA clones that encode DRE binding proteins, DREB1A and DREB2A, were isolated by using the yeast one-hybrid screening technique. The two cDNA libraries were prepared from dehydrated and cold-treated rosette plants, respectively. The deduced amino acid sequences of DREB1A and DREB2A showed no significant sequence similarity, except in the conserved DNA binding domains found in the EREBP and APETALA2 proteins that function in ethylene-responsive expression and floral morphogenesis, respectively. Both the DREB1A and DREB2A proteins specifically bound to the DRE sequence in vitro and activated the transcription of the β-glucuronidase reporter gene driven by the DRE sequence in Arabidopsis leaf protoplasts. Expression of the *DREB1A* gene and its two homologs was induced by low-temperature stress, whereas expression of the *DREB2A* gene and its single homolog was induced by dehydration. Overexpression of the DREB1A cDNA in transgenic Arabidopsis plants not only induced strong expression of the target genes under unstressed conditions but also caused dwarfed phenotypes in the transgenic plants. These transgenic plants also revealed freezing and dehydration tolerance. In contrast, overexpression of the DREB2A cDNA induced weak expression of the target genes under unstressed conditions and caused growth retardation of the transgenic plants. These results indicate that two independent families of DREB proteins, DREB1 and DREB2, function as *trans*-acting factors in two separate signal transduction pathways under low-temperature and dehydration conditions, respectively.

INTRODUCTION

Drought and low temperature are adverse environmental conditions that affect the growth of plants and the productivity of crops. However, it has been suggested that plants have common mechanisms in their physiological responses and tolerance to drought and low temperature. For example, abscisic acid (ABA) is produced under both drought and low-temperature stresses and plays important roles in allowing plants to tolerate both stresses. Also, plants grown under dehydration conditions show higher tolerance to low-temperature stress than do well-watered plants.

A number of genes have been described that respond to both drought and low-temperature stress at the transcriptional level (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). The functions of some gene products have been predicted from sequence homology with known proteins and are thought to play a role in protecting the cells from water deficit and low temperature (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). Most of the drought- and cold stress–inducible genes that have been studied to date are also induced by ABA. Dehydration appears to trigger the production of ABA, which in turn induces expression of various

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genes. *cis*- and *trans*-acting factors involved in ABA-induced gene expression have been analyzed extensively (reviewed in Chandler and Robertson, 1994; Giraudat et al., 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997).

However, expression of several ABA-inducible genes is induced by both cold and drought in ABA-deficient (*aba*) and ABA-insensitive (*abi*) Arabidopsis mutants. This suggests that these genes do not require ABA for their expression under cold and drought conditions but do respond to ABA (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). These genes include *rd29A*/*lti78*/*cor78*, *kin1*, *cor6.6*/*kin2*, and *cor47*/*rd17* (Nordin et al., 1991; Kurkela and Borg-Franck, 1992; Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; Iwasaki et al., 1997). The promoter region of the *rd29A* gene was analyzed, and a novel *cis*-acting element responsible for dehydration- and cold-induced expression was identified at the nucleotide sequence level by using transgenic plants (Yamaguchi-Shinozaki and Shinozaki, 1994). A 9-bp conserved sequence, TACCGACAT, termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression. The DRE has been demonstrated to function as a *cis*-acting element involved in the induction of *rd29A* expression by low-temperature stress.

DRE-related motifs have been reported in the promoter regions of cold- and drought-inducible genes such as *kin1*, *cor6.6*, and *rd17* (Wang et al., 1995; Iwasaki et al., 1997). A similar motif was also reported (C repeat; TGGCCGAC) in the promoter region of cold-inducible *cor15a* (Baker et al., 1994). The CCGAC core sequence was found in the promoter regions of the cold-inducible oilseed rape gene *BN115* and designated the low-temperature-responsive element (Jiang et al., 1996). These results suggest that DRE-related motifs are involved in both drought- and cold-responsive but ABA-independent gene expression.

It is important to understand how two different stress signals, drought and cold, are transmitted separately in plant cells to activate DRE-dependent transcription of the *rd29A*/*cor78* gene. For this purpose, it is critical to identify *trans*-acting factors that regulate DRE-dependent gene expression. We attempted to isolate cDNAs for DRE binding proteins by using the DNA ligand binding screening method, but we were not successful. Next, we tried to isolate cDNAs for DRE binding proteins by using the yeast one-hybrid screening system. Meanwhile, Stockinger et al. (1997) reported cloning a cDNA (named CBF1) for a C repeat/DRE binding protein from Arabidopsis by using yeast one-hybrid screening. The CBF1 protein has a DNA binding motif found in tobacco EREBP1 (Ohme-Takagi and Shinshi, 1995), which is involved in ethylene-responsive gene expression, and in Arabidopsis APETALA2 (AP2; Jofuku et al., 1994), which is involved in floral morphogenesis. The CBF1 protein can bind to the C repeat/DRE motif in the *cor15a* promoter and function as a *trans*-activator in yeast. However, the CBF1 cDNA clone was isolated from a cDNA library prepared from unstressed normally grown Arabidopsis plants, and it had an abnormally fused structure with the 25S rRNA gene (Stockinger et al., 1997).

In contrast, we cloned two different cDNAs encoding DRE binding proteins (DREB1A and DREB2A) of Arabidopsis that specifically interact with the DRE sequence in the promoter region of the *rd29A* gene from dehydrated and low-temperature-treated Arabidopsis plants by using the yeast one-hybrid screening method. Genes encoding the DREB1A protein and its two homologs were induced to express by cold stress; genes encoding the DREB2A protein and its single homolog rapidly were induced to express by dehydration and high-salt stress. Both the DREB1A and DREB2A homologs contain the EREBP/AP2 DNA binding domain like that of CBF1. We analyzed the function of the DREB1A and DREB2A proteins as *trans*-acting factors by using transient expression in Arabidopsis leaf protoplasts and overexpression in transgenic Arabidopsis plants. We discuss the different functions of the DREB1A and DREB2A proteins in the separation of two signaling pathways under cold and dehydration stress conditions in ABA-independent gene expression in vegetative tissues.

RESULTS

Isolation of cDNAs Encoding DNA Binding Proteins That Recognize DRE in the 71-bp DNA Fragment of the *rd29A* Promoter

To isolate cDNAs encoding DNA binding proteins that interact with the DRE motif, we used the yeast one-hybrid screening system. We first constructed a parental yeast strain carrying as dual reporter genes integrated copies of *HIS3* and *lacZ* with four-times tandemly repeated 71-bp DNA fragments of the *rd29A* promoter upstream of the TATA element (Figure 1). The 71-bp fragment contains a DRE motif at center. The resulting yeast strain transcribes the *HIS3* gene at basal levels, grows on media lacking histidine (but not in the presence of 10 mM 3-aminotriazole [3-AT], a competitive inhibitor of the *HIS3* gene product), and forms white colonies on filter papers containing X-gal. The yeast cells were then separately transformed with three expression libraries of cDNA fragments of mRNAs prepared from Arabidopsis rosette plants that had been dehydrated for 2 hr, cold treated for 24 hr, or undehydrated. The cDNA fragments were fused to the transcriptional activation domain of the yeast GAL4 (Figure 1).

We screened 1.8×10^6, 1.2×10^6, and 1.5×10^6 yeast transformants of libraries prepared from 2-hr dehydrated, 24-hr cold-treated, and unstressed Arabidopsis rosette plants, respectively. Clones 2 to 41 and clone 1 were 3-AT resistant and isolated from libraries prepared from 2-hr dehydrated and 24-hr cold-treated plants, respectively (Table 1). All of the isolated cDNA clones induced *lacZ* activity and formed blue colonies on filter papers containing X-gal. The

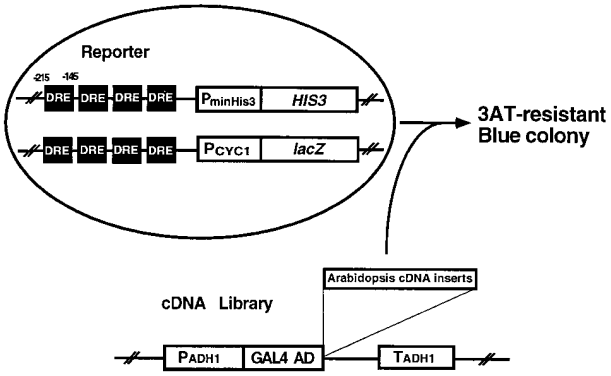

Figure 1. Strategy for the Isolation of cDNAs Encoding DRE Binding Proteins by Selection in Yeast.

An expression library of hybrid proteins was transformed into the yeast strain carrying dual reporter genes *HIS3* and *lacZ* under the control of the 71-bp promoter region of *rd29A* containing the DRE. The hybrids contain protein coding sequences fused to the end of the GAL4 activation domain (AD). Hybrid proteins that recognize the binding site act as transcriptional activators of the reporter genes, allow the cells to grow in the presence of 3-AT (a competitive inhibitor of the *HIS3* gene product), and turn the cells blue in β-galactosidase assay. $P_{minHis3}$ indicates the minimal promoter of the *HIS3* gene, and P_{CYC1} indicates the minimal promoter of the yeast cyclin gene. PADH1 indicates the promoter of the alcohol dehydrogenase1 (*ADH1*) gene, and TADH1 indicates the terminator of the *ADH1* gene.

cDNA fragments of the isolated plasmids were analyzed by restriction enzyme digestion and DNA sequencing, which led to the classification of these 41 cDNA clones into seven distinct cDNA groups. Among the seven groups, clone 18 was most abundant in the cDNA library prepared from dehydrated plants (Table 1).

To select cDNAs that encode transcriptional activators in the seven independent cDNA clones that were isolated, the insert cDNA fragments were cloned into the yeast expression vector YepGAP (Figure 2A). Plasmids containing each insert DNA fragment were transformed into yeast strains carrying the dual reporter genes *HIS3* and *lacZ* that had been fused to 71-bp DNA fragments of the *rd29A* promoter containing the DRE sequence. Yeast cells carrying the plasmid containing the cDNA inserts of clones 1 and 18 grew on medium lacking histidine in the presence of 10 mM 3-AT, but yeast cells carrying the plasmid containing the cDNA inserts of five other clones did not. Both the 3-AT–resistant yeast strains also induced *lacZ* activity and formed blue colonies (Figure 2B). In contrast, when plasmids containing the DNA insert of clone 1 or 18 were transformed into yeast strains carrying the dual reporter genes fused to the 71-bp DNA fragment with base substitutions in the DRE sequence mDRE, the yeast strains neither grew on media lacking histidine in the presence of 10 mM 3-AT nor induced *lacZ* activity (Figure 2C). These data indicate that cDNA clones 1 and 18 encode polypeptides that specifically bind to the DRE sequence and activate the transcription of the dual reporter genes in yeast. cDNA clones 1 and 18 were designated DREB1A and DREB2A, respectively, and analyzed further.

Structural Analysis of the DREB1A and DREB2A cDNAs

To examine the structures of the DREB1A and DREB2A cDNA clones, we sequenced inserted DNA fragments of 0.9 and 1.4 kb, respectively. The DREB1A cDNA contains a single open reading frame of 216 amino acids and encodes a putative protein with a predicted molecular mass of 24.2 kD (Figure 3). The DREB2A cDNA contains an open reading frame of 335 amino acids and encodes a putative protein with a predicted molecular mass of 37.7 kD (Figure 3).

We searched DNA and protein databases for sequences homologous to those of the DREB1A and DREB2A proteins and found that each DREB protein has a conserved DNA binding domain of 58 amino acids present in a large family of plant genes for DNA binding proteins, including EREBPs of tobacco and AP2 of Arabidopsis (Figure 4). The deduced amino acid sequences of DREB1A and DREB2A showed no significant sequence identity except in the conserved DNA binding domain. However, each DREB protein contains a basic region in its N-terminal region that might function as a nuclear localization signal and an acidic C-terminal region that might act as an activation domain for transcription. These data suggest that each DREB cDNA encodes a DNA binding protein that might function as a transcriptional activator in plants.

DNA Binding Regions of the DREB1A and DREB2A Proteins Bind Specifically to the DRE Sequence of the *rd29A* Promoter

To identify the target sequence of the DREB1A and DREB2A proteins, the 143 and 166 amino acids of the DNA binding

Table 1. General Characteristics of the cDNA Clones Isolated in This Study[a]

Group	Clone	Insert Size (kb)	No. of Clones Obtained
1	1	0.94	1
2	18	1.4	35
3	63	1.8	1
4	75	0.48	1
5	104	0.77	1
6	125	0.48	1
7	127	1.2	1

[a] Forty-one positive clones were isolated from libraries prepared from dehydrated and cold-treated plants. These 41 cDNA clones were divided into seven distinct cDNA groups.

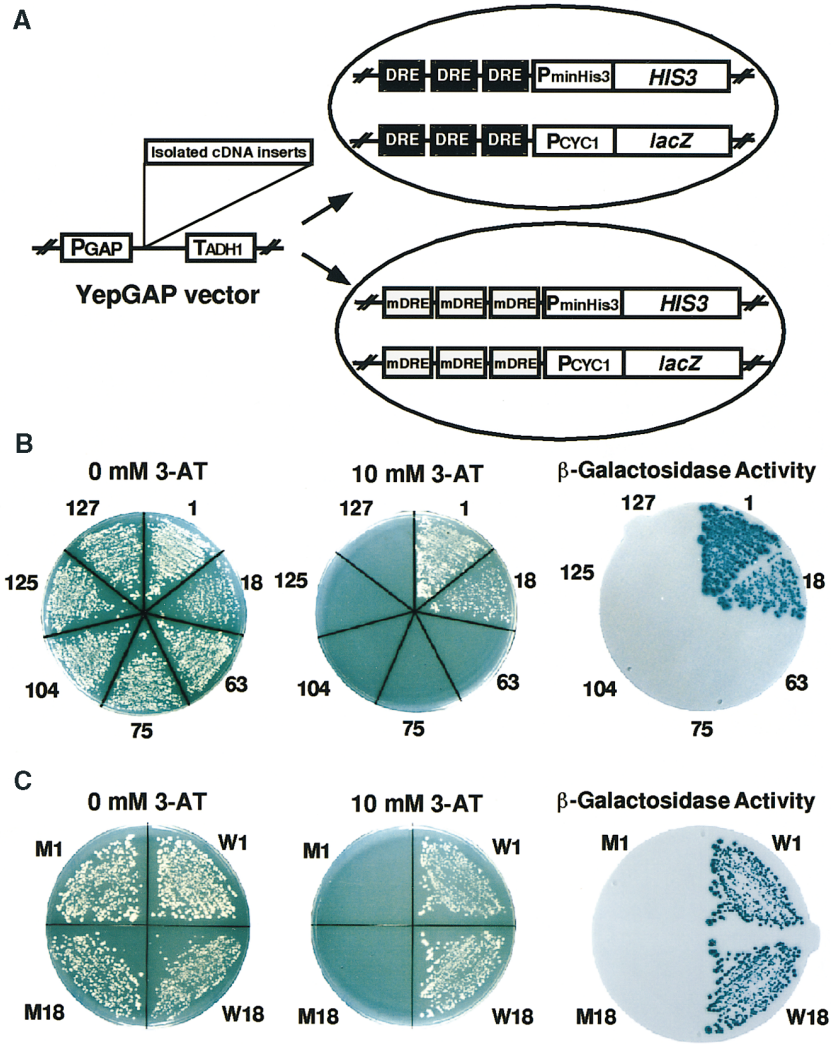

Figure 2. Activation of Dual Reporter Genes in Yeast by Proteins Encoded by Isolated cDNAs.

(A) The insert DNA fragments of the isolated cDNA clones were cloned into the yeast expression vector YepGAP and used for transformation into yeast carrying the dual reporter genes *HIS3* and *lacZ* under the control of the 71-bp promoter region containing the DRE or the 71-bp promoter region containing a mutated DRE (mDRE; M2 in Figure 5). Other abbreviations are as given in the legend to Figure 1. P_{GAP} indicates the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene. TADH1 indicates the terminator of the *ADH1* gene.
(B) All of the yeast transformants carrying dual reporter genes under the control of the wild-type 71-bp promoter region were examined for growth in the presence of 3-AT and β-galactosidase activity. Numbers indicate isolated clone names.
(C) Two plasmids containing insert DNA from clones 1 and 18 were transformed into yeast strains carrying the dual reporter genes under the control of the 71-bp promoter region containing the DRE (W1 and W18) or the mutated DRE sequence (M1 and M18). The transformants were examined for growth in the presence of 3-AT and β-galactosidase activity.

domains of DREB1A and DREB2A, respectively, were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli.* The ability of the DREB1A and DREB2A fusion proteins to bind the wild-type or mutated DRE sequences was examined using the gel retardation method. As shown in Figure 5B, both the recombinant DREB1A and DREB2A fusion proteins bound the wild-type 71-bp DNA fragment but not the base-substituted 71-bp fragments M1, M2, and M3. By contrast, both of the fusion proteins bound to the base-substituted 71-bp fragments M4 and M5. The DRE sequence was base-substituted in M1, M2, and M3 but not in M4 and M5 (Figure 5A). These results indicate that the binding of the DREB1A and DREB2A fusion proteins to the DRE sequence is highly specific.

Expression of the *DREB1A* and *DREB2A* Genes

The expression patterns of the *DREB1A* and *DREB2A* genes were analyzed using RNA gel blot hybridization to compare them with that of the *rd29A* gene (Figure 6A). *DREB2A* gene expression was induced within 10 min after dehydration began, and *DREB2A* was strongly expressed after 2 hr. However, there was no significant *DREB1A* mRNA accumulation within 24 hr. There was significant *DREB2A* mRNA accumulation within 10 min after high-salt treatment, whereas the *DREB1A* mRNA was not accumulated (Figure 6A). When, as a control, the plants were transferred from agar to water, rapid but low-level accumulation of *DREB2A* mRNA was detected, whereas *DREB1A* mRNA accumulation was not apparent. Similar results were obtained for the exogenous ABA treatment. By contrast, the *DREB1A* gene was induced to express within 1 hr after exposure to low temperature (4°C), and the level of the *DREB1A* mRNA peaked after 2 hr. However, *DREB2A* mRNA did not accumulate significantly within 24 hr after exposure to low temperature (Figure 6A). These results indicate that the transcription of the *DREB1A* gene is activated by cold stress and that that of the *DREB2A* gene is activated by dehydration and high-salt stress.

Expression of the *rd29A* gene was induced within 20 min by dehydration, and the gene was strongly expressed after 2 hr (Figure 6A). The *rd29A* gene also was induced to express by low-temperature treatment within 2 hr and was strongly expressed after 5 hr. Rapid and strong expression of the *rd29A* gene was observed within 10 min after the initiation of ABA treatment as well as by high-salt treatment. When plants were transferred from agar to water, rapid but weak expression of the *rd29A* gene was detected. The expression of the *DREB1A* gene during cold stress preceded that of the *rd29A* gene (Figure 6A). In contrast, the expression pattern of the *DREB2A* gene during dehydration and high-salt stress was similar to that of the *rd29A* gene. We could detect both *DREB2A* mRNA and *rd29A* mRNA but not *DREB1A* mRNA in all of the tissues of unstressed plants (Figure 6B).

DREB1A

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CCTGAACTAGAACAGAAAGAGAGAGAAACTATTATTTCAGCAAACCATACCAACAAAAAAGACAGAGATCTTTTA   75
GTTACCTTATCCAGTTTCTTGAAACAGAGTACTCTTCTGATCAATGAACTCATTTTCTGCTTTTCTGAAATGTT  150
                                        M  N  S  F  S  A  F  S  E  M  F
TGGCTCCGATTACGAGTCTTCGGTTTCCTCAGGCCGTGATTATATTCCGACGCTTGCGAGCAGCTGCCCCAAGAA  225
 G  S  D  Y  E  S  S  V  S  S  G  G  D  Y  I  P  T  L  A  S  S  C  P  K  K
ACCGGCGGGTCGTAAGAAGTTTCGTGAGACTCGTCACCCAATATACAGAGGAGTTCGTCGGAGAAACTCCGGTAA  300
 P  A  G  R  K  K  F  R  E  T  R  H  P  I  Y  R  G  V  R  R  R  N  S  G  K
GTGGGTTTGTGAGGTTAGAGAACCAAACAAGAAAACAAGGATTTGGCTCGGAACATTTCAAACCGCTGAGATGCC  375
 W  V  C  E  V  R  E  P  N  K  K  T  R  I  W  L  G  T  F  Q  T  A  E  M  A
AGCTCGAGCTCACGACGTTGCCGCTTTAGCCCTTCGTGGCCGATCAGCCTGTCTCAATTTCGCTGACTCGGCTTG  450
 A  R  A  H  D  V  A  A  L  A  L  R  G  R  S  A  C  L  N  F  A  D  S  A  W
GAGACTCCGAATCCCGGAATCAACTTGCGCTAAGGACATCCAAAAGGCGGCGGCTGAAGCTGCGTTGGCGTTTCA  525
 R  L  R  I  P  E  S  T  C  A  K  D  I  Q  K  A  A  A  E  A  A  L  A  F  Q
GGATGAGATGTGTGATGCGACGACGGATCATGGCTTCGACATGGAGGAGACGTTGGTGGAGGCTATTTACACGGC  600
 D  E  M  C  D  A  T  T  D  H  G  F  D  M  E  E  T  L  V  E  A  I  Y  T  A
GGAACAGAGCGAAAATGCGTTTTATATGCACGATGAGGCGATGTTTGAGATGCCGAGTTTGTTGCTGGCTAATATGGC  675
 E  Q  S  E  N  A  F  Y  M  H  D  E  A  M  F  E  M  P  S  L  L  A  N  M  A
AGAAGGGATGCTTTTTGCCGCTTCCGTCCGTACAGTGGAATCATAATCATGAAGTCGACGGCGATGATGACGACGT  750
 E  G  M  L  L  P  L  P  S  V  Q  W  N  H  N  H  E  V  D  G  D  D  D  D  V
ATCGTTATGGAGTTATTAAAACTCAGATTATTATTTCCATTTTTAGTACGATACTTTTTATTTTATTATTATTTTT  825
 S  L  W  S  Y
TAGATCCTTTTTTAGAATGGAATCTTCATTATGTTTGTAAAACTGAGAAACGAGTGTAAATTAAATTGATTCAGT  900
TTCAGTATAAAAAAAAAAAAAAAAAAAAAAAA                                              933
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DREB2A

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GCTGTCTGATAAAAAGAAGAGGAAAACTCGAAAAAGCTACACACAAGAAGAAGAAGAAAAGATACGAGCAAGAAG   75
ACTAAACACGAAAGCGATTTATCAACTCGAAGGAAGAGACTTTGATTTTCAAATTTCGTCCCCTATAGATTGTGT  150
TGTTTCTGGGAAGGAGATGGCAGTTTATGATCAGAGTGGAGATAGAAACAGAACACAAATTGATACATCGAGGAA  225
                  M  A  V  Y  D  Q  S  G  D  R  N  R  T  Q  I  D  T  S  R  K
AAGGAAATCTAGAAGTAGAGGTGACGGTACTACTGTGGCTGAGAGATTAAAGAGATGGAAAGATGTATAACGAGAC  300
 R  K  S  R  S  R  G  D  G  T  T  V  A  E  R  L  K  R  W  K  E  Y  N  E  T
CGTAGAAGAAGTTTCTACCAAGAAGAGGAAAGTACCTGCGAAAGGGTCGAAGAAGGGTTGTATGAAAGGTAAAGG  375
 V  E  E  V  S  T  K  K  R  K  V  P  A  K  G  S  K  K  G  C  M  K  G  K  G
AGGACCAGAGAATAGCCGATGTAGTTTCAGAGGAGTTAGGCAAAGGATTTGGGGTAAATGGGTTGCTGAGATCAG  450
 G  P  E  N  S  R  C  S  F  R  G  V  R  Q  R  I  W  G  K  W  V  A  E  I  R
AGAGCCTAATCGAGGTAGCAGGCTTTGGCTTGGTACTTTCCCTACTGCTCAAGAAGCTGCTTCTGCTTATGATGA  525
 E  P  N  R  G  S  R  L  W  L  G  T  F  P  T  A  Q  E  A  A  S  A  Y  D  E
GGCTGCTAAAGCTATGTATGGTCCTTTGGCTCGTCTTAATTTCCCTCGGTCTGATGCGTCTGAGGTTACGAGTAC  600
 A  A  K  A  M  Y  G  P  L  A  R  L  N  F  P  R  S  D  A  S  E  V  T  S  T
CTCAAGTCAGTCTGAGGTGTGTACTGTTGAGACTCCTGGTTGTGTTCATGTGAAAACAGAGGATCCAGATTGTGA  675
 S  S  Q  S  E  V  C  T  V  E  T  P  G  C  V  H  V  K  T  E  D  P  D  C  E
ATCTAAACCCTTCTCCGGTGGAGTGGAGCCGATGTATTGTCTGGAGAATGGTGCGGAAGAGATGAAGAGAGGTGT  750
 S  K  P  F  S  G  G  V  E  P  M  Y  C  L  E  N  G  A  E  E  M  K  R  G  V
TAAAGCGGATAAGCATTGGCTGAGCGAGTTTGAACATAACTATTGGAGTGATATTCTGAAAGAGAAAGAGAAACA  825
 K  A  D  K  H  W  L  S  E  F  E  H  N  Y  W  S  D  I  L  K  E  K  E  K  Q
GAAGGAGCAAGGGATTGTAGAAACCTGTCAGCAACAACAGCAGGATTCGCTATCTGTTGCAGACTATGGTTGGCC  900
 K  E  Q  G  I  V  E  T  C  Q  Q  Q  Q  Q  D  S  L  S  V  A  D  Y  G  W  P
CAATGATGTGGATCAGAGTCACTTGGATTCTTCAGACATGTTTGATGTCGATGAGCTTCTACGTGACCTAAATGG  975
 N  D  V  D  Q  S  H  L  D  S  S  D  M  F  D  V  D  E  L  L  R  D  L  N  G
CGACGATGTGTTTGCAGGCTTAAATCAGGACCGGTACCCGGGGAACAGTGTTGCCAACGGTTCATACAGGCCCGA 1050
 D  D  V  F  A  G  L  N  Q  D  R  Y  P  G  N  S  V  A  N  G  S  Y  R  P  E
GAGTCAACAAAGTGGTTTTGATCCGCTACAAAGCCTCAACTACGGAATACCTCCGTTTCAGCTCGAGGGAAAGGA 1125
 S  Q  Q  S  G  F  D  P  L  Q  S  L  N  Y  G  I  P  P  F  Q  L  E  G  K  D
TGGTAATGGATTCTTCGACGACTTGAGTTACTTGGATCTGGAGAACTAAACAAAACAATATGAAGCTTTCTACGTGACCTAAATGG 1200
 G  N  G  F  F  D  D  L  S  Y  L  D  L  E  N
TTGATATTTGCCTTAATCCCACAACGACTGTTGATTCTCTATCCGAGTTTTAGTGATATAGAGAACTACAGAACA 1275
CGTTTTTTCTTGTTATAAAGGTGAACTGTATATATCGAAACAGTGATATGACAATAGAGAAGACAACTATAGTTT 1350
GTTAGTCTGCTTCTCTTAAGTTGTTCTTTAGATATGTTTTATGTTTTGTAACAACAGGAATGAATAATACACACT 1425
TGTAAAAAAAAA                                                                  1437
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Figure 3. Full-Length Sequences of DREB1A and DREB2A cDNAs.

The EREBP/AP2 domains are underlined. Each DREB protein contains a basic region in the N-terminal region that might function as a nuclear localization signal (double underlines) and an acidic C-terminal region that might act as a transcriptional activation domain. The DREB1A and DREB2A cDNA sequences have been submitted to the GenBank, EMBL, and DDBJ data bases with accession numbers AB007787 and AB007790, respectively.

Isolation of cDNAs That Encode Homologs of the DREB1A and DREB2A Proteins

The number of DREB-related genes in the Arabidopsis genome was estimated by DNA gel blot analysis (Figure 7). Nuclear DNA from Arabidopsis was digested with BamHI, EcoRI, and HindIII and hybridized under both high- and low-stringency conditions by using the DREB1A and DREB2A cDNA inserts as probes. Under high-stringency hybridization conditions, each probe hybridized with a few bands of DNA fragments. Under low-stringency conditions, a few additional bands were detected, suggesting that there may be a few DREB-related genes in the Arabidopsis genome.

To isolate cDNAs for the DREB-related genes, three λgt11 cDNA libraries prepared from dehydrated, cold-treated, and unstressed plants, respectively, were screened with the DREB1A and DREB2A cDNA inserts as probes. Three independent cDNA clones were isolated using the DREB1A cDNA as a probe; one is identical to DREB1A and the other two are homologs (named DREB1B and DREB1C). The DREB1B clone is identical to CBF1 (Stockinger et al., 1997). The DREB1C cDNA contains a single open reading frame of 216 amino acids and encodes a putative protein with a predicted molecular mass of 24.3 kD (Figure 8A). The three DREB1A homologs have highly homologous amino acid sequence identity (Figure 8A, DREB1A and DREB1B, 86%; DREB1B and DREB1C, 86%; DREB1A and DREB1C, 87%). Moreover, the low-temperature-inducible expression of the *DREB1B* and the *DREB1C* genes was also similar to that of the *DREB1A* gene (data not shown).

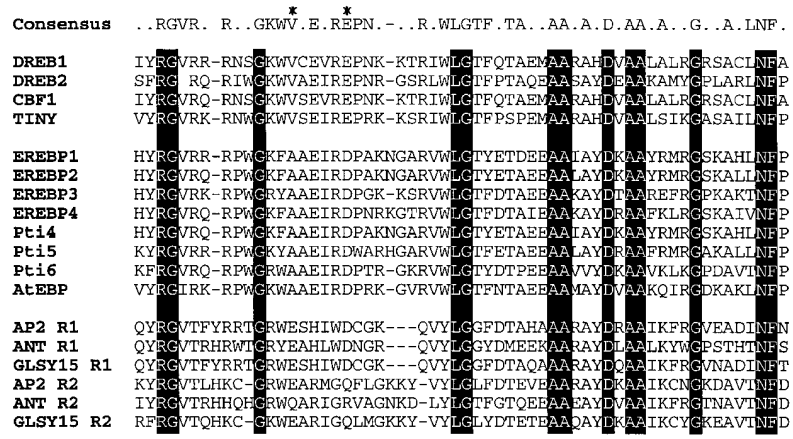

Figure 4. Comparison of Deduced Amino Acid Sequences of the DNA Binding Domains of DREB1A and DREB2A with Those of Other EREBP/AP2–Related Proteins.

The deduced amino acid sequences of DREB1A and DREB2A are compared with EREBP/AP2–related proteins, namely, Arabidopsis CBF1 (Stockinger et al., 1997), Arabidopsis TINY (Wilson et al., 1996), tobacco EREBP1 to EREBP4 (Ohme-Takagi and Shinshi, 1995), tomato Pti4 to Pti6 (Zhou et al., 1997), Arabidopsis AtEBP1 (AtEBP; Buttner and Singh, 1997), Arabidopsis AP2 (Jofuku et al., 1994), Arabidopsis AINTEGUMENTA (ANT; Klucher et al., 1996), and maize GLOSSY15 (GLSY15; Moose and Sisco, 1996). The black background represents perfectly conserved amino acid residues, and dashes indicate gaps introduced to maximize alignment. Consensus indicates the conserved amino acids in DREB1A and DREB2A. Asterisks represent different amino acids in the consensus between DRE binding proteins and GCC box binding proteins. R1 and R2 indicate repeated amino acid sequences of the EREBP/AP2 motif.

No DREB2A homolog was isolated by screening cDNA libraries by using the DREB2A cDNA as a probe. Arabidopsis cDNA libraries were then screened using the yeast one-hybrid system. We screened 7.6×10^6, 7.4×10^6, and 7.8×10^6 yeast transformants of the libraries prepared from 2-hr dehydrated, 24-hr cold-treated, and unstressed Arabidopsis rosette plants, respectively. We isolated cDNA clones encoding a DREB2A homolog from the library prepared from 2-hr dehydrated plants (named DREB2B; Figure 8B). The DREB2B cDNA contains a single open reading frame of 330 amino acids and encodes a putative protein with a predicted molecular mass of 37.1 kD (Figure 8B). DREB2A and DREB2B show sequence similarity (Figure 8B; 53.8%), especially in the N-terminal region. A serine- and threonine-rich region following the DNA binding domain and a glutamine-rich region in the C-terminal region were found in both the DREB2A and DREB2B proteins (Figure 8B). The dehydration-induced and high-salinity-induced expression of the *DREB2B* gene was similar to that of the *DREB2A* gene (data not shown). These observations indicate that the two *DREB2* genes are clearly different from the three *DREB1* genes.

DREB1A and DREB2A Proteins Transactivate the *rd29A* Promoter–*GUS* Fusion Gene in Leaf Protoplasts

To determine whether the DREB1A and DREB2A proteins are capable of transactivating DRE-dependent transcription in plant cells, we performed transactivation experiments using protoplasts prepared from Arabidopsis leaves. Protoplasts were cotransfected with a β-glucuronidase (*GUS*) reporter gene fused to the trimeric 71-bp fragments containing the DRE motif and the effector plasmid (Figure 9A). The effector plasmid consisted of the cauliflower mosaic virus (CaMV) 35S promoter fused to DREB1A or DREB2A cDNAs. The tobacco mosaic virus (TMV) Ω sequence was inserted upstream from these cDNAs to strengthen their translation efficiency. Coexpression of the DREB1A or DREB2A proteins in protoplasts transactivated the expression of the *GUS* reporter gene (Figure 9B). These results suggest that DREB1A and DREB2A proteins function as transcription activators involved in the cold- and dehydration-responsive expressions, respectively, of the *rd29A* gene.

Analysis of the in Vivo Roles of DREB1A and DREB2A in Expression of the *rd29A* Gene by Using Transgenic Plants

To analyze the effects of overproduction of DREB1A and DREB2A proteins on the expression of the *rd29A* gene, we generated transgenic plants in which DREB1A or DREB2A cDNAs were introduced to overproduce DREB proteins. Arabidopsis plants were transformed with binary vectors carrying fusions of the enhanced CaMV 35S promoter (Mituhara et al., 1996) and the DREB1A (35S:DREB1A) or DREB2A (35S:DREB2A) cDNAs in the sense orientation. The TMV Ω sequence (Gallie et al., 1987) was inserted upstream from

these cDNAs to strengthen their translation level. Eighteen and eight antibiotic-resistant Arabidopsis transformants for DREB1A and DREB2A, respectively, were generated by using a vacuum infiltration method (Bechtold et al., 1993). Transgenic plants of the T_2 generation were used for further analyses.

All of the 18 plants carrying the 35S:DREB1A transgene (the 35S:DREB1A plants) had dwarf phenotypes under normal growth conditions. The 35S:DREB1A plants showed variations in phenotypic changes in growth retardation that may have been due to the different levels of expression of the DREB1A transgenes for the position effect. Three different phenotypic changes in growth of the 35S:DREB1A plants were compared with wild-type plants (Figure 10). Three of the 18 35S:DREB1A plants, including the 35S:DREB1Aa plants, showed severe dwarf phenotypes, whereas the others revealed growth retardation as shown by the results with the 35S:DREB1Ab and 35S:DREB1Ac plants (Figure 10). In the severely dwarfed 35S:DREB1Aa plants, the DREB1A transcript accumulated to a high level under the unstressed control condition. The higher level of the DREB1A transcripts in the 35S:DREB1A plants caused the more severe dwarf phenotypes of the transgenic plants (Figure 11).

To analyze whether overproduction of the DREB1A protein caused the expression of the target gene in unstressed plants, we compared the expression of the *rd29A* gene in control plants carrying pBI121 vector (wild type) with that in the 35S:DREB1A plants. Transcription of the *rd29A* gene was low in the unstressed wild-type plants but high in the unstressed 35S:DREB1A plants. The level of the *rd29A* transcripts under the unstressed control condition was found to depend on the level of the DREB1A transcripts. Expression of the *rd29A* gene was induced by dehydration, high salt and cold stress, and ABA treatment in the 35S:DREB1A plants as well as in wild-type plants. However, the level of the *rd29A* transcripts in the 35S:DREB1A plants was higher than that in the wild-type plants, even under stressed conditions (Figure 11).

The transgenic plants carrying the 35S:DREB2A transgene (the 35S:DREB2A plants) showed little phenotypic change. However, 35S:DREB2Aa plants exhibited slight growth retardation (Figure 10). The level of *DREB2A* mRNA was higher in 35S:DREB2Aa plants than in the normal 35S:DREB2Ab plants (Figure 11). Expression of the *rd29A* gene in the 35S:DREB2Aa plants having little phenotypic change under unstressed conditions was slightly higher than in the normal 35S:DREB2Ab plants (Figure 11).

Freezing and Dehydration Tolerance of Transgenic Plants

The tolerance to freezing and dehydration of the transgenic plants was analyzed using the 35S:DREB1Ab and 35S:DREB1Ac plants grown in pots at 22°C for 3 weeks. When plants grown in pots were exposed to a temperature of −6°C for 2 days, returned to 22°C, and grown for 5 days,

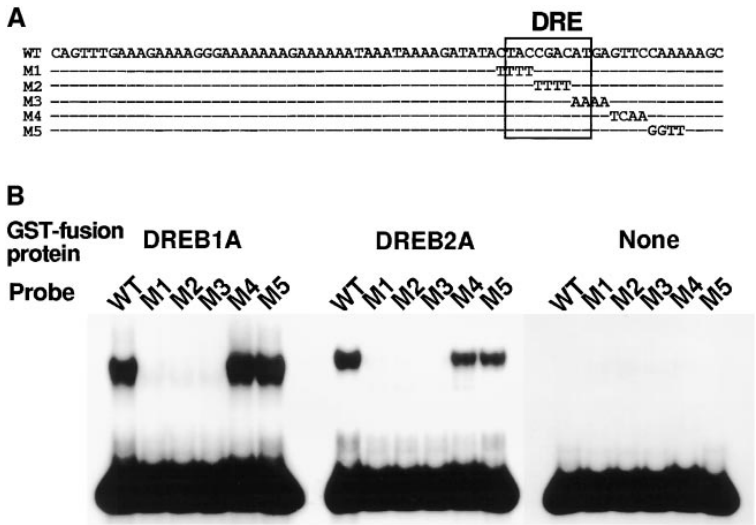

Figure 5. Characterization of the DNA Binding Affinity of the Recombinant DREB1A and DREB2A Proteins to the 71-bp Fragment (Positions −215 to −145) of the *rd29A* Promoter.

(A) Upper strand sequence of the 71-bp fragment of the *rd29A* promoter (WT) and its mutated fragments (M1 to M5) used as probes.
(B) Gel retardation assay of sequence-specific binding of the recombinant DREB1A and DREB2A proteins. The radioactive probes were incubated in the presence or absence (None) of the recombinant DREB1A or DREB2A proteins.

all of the wild-type plants died, whereas the 35S:DREB1Ab plants survived at high frequency (83.9% survival; Figure 12). The surviving plants continued to grow and flowered under unstressed conditions. Freezing tolerance was correlated with the level of expression of the stress-inducible genes under unstressed control conditions. The 35S:DREB1Ab plants with high-level expression of the target genes showed higher freezing tolerance than did the 35S:DREB1Ac plants with their low-level expression (35.7% survival; Figure 12).

To test whether the introduction of the DREB1A gene enhances tolerance to dehydration stress, for 2 weeks we did not water the wild-type and transgenic plants grown in pots (Figure 12). Although all of the wild-type plants died within 2 weeks, 42.9% of the 35S:DREB1Ab plants survived and continued to grow after rewatering. Drought tolerance was also dependent on the level of expression of the target genes in the 35S:DREB1A plants under unstressed conditions. The survival rate of the 35S:DREB1Ac plants was lower than that of the 35S:DREB1Ab plants (21.4% survival; Figure 12).

DISCUSSION

Using the yeast one-hybrid screening system, we identified two distinct cDNAs, DREB1A and DREB2A, encoding DNA binding proteins that specifically interact with the DRE sequence involved in dehydration-, high-salt-, and low-temperature-responsive gene expression. The DREB1A and

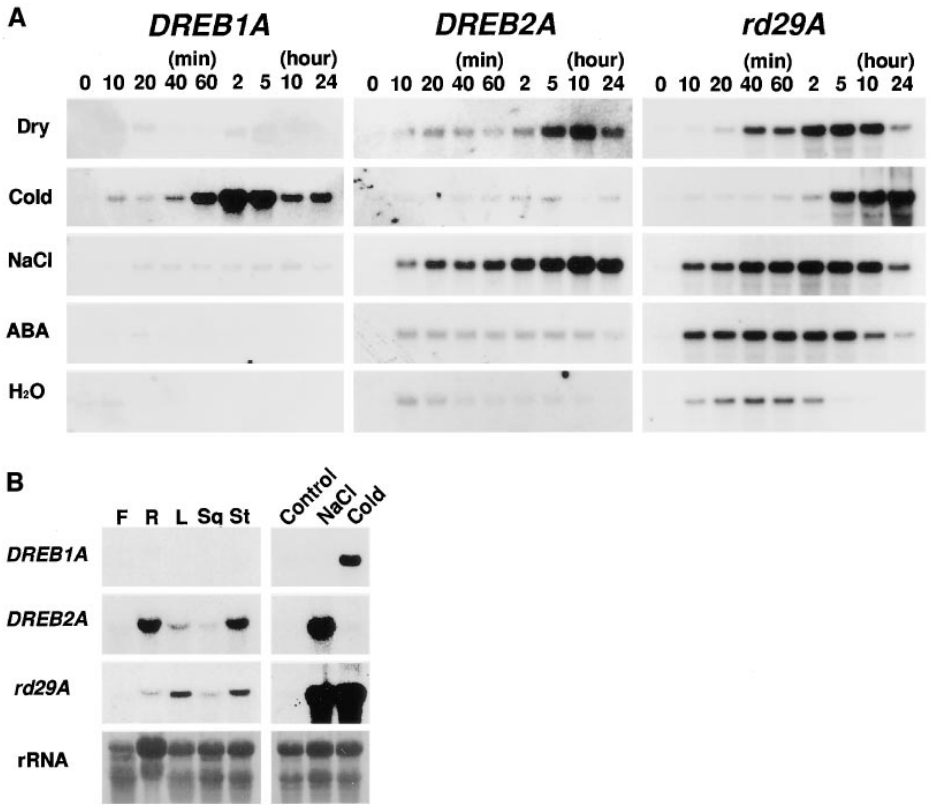

Figure 6. RNA Gel Blot Analysis of DREB1A and DREB2A Transcripts.

(A) Expression of the *DREB1A*, *DREB2A*, and *rd29A* genes in response to dehydration, low temperature, high salt, or ABA. Each lane was loaded with 20 μg of total RNA from 3-week-old unbolted Arabidopsis plants that had been dehydrated (Dry), transferred to and grown at 4°C (Cold), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), transferred from agar plates to hydroponic growth in 100 μM ABA (ABA), or transferred from agar plates to water (H_2O) for hydroponic growth, as described in Methods. The number above each lane indicates the number of minutes or hours after the initiation of treatment before isolation of RNA. RNA was analyzed by RNA gel blotting, with gene-specific probes from the 3′ flanking sequences of *DREB1A*, *DREB2A*, and *rd29A*.

(B) Expression of the *DREB1A*, *DREB2A*, and *rd29A* genes in a variety of organs of normally grown Arabidopsis. Each lane was loaded with 40 μg of total RNA prepared from flowers (F), roots (R), leaves (L), siliques (Sq), stems (St), whole plants (Control), whole plants treated with 250 mM NaCl for 5 hr (NaCl), and whole plants cold-treated at 4°C for 5 hr (Cold). rRNAs blotted on the membrane were visualized by staining with methylene blue.

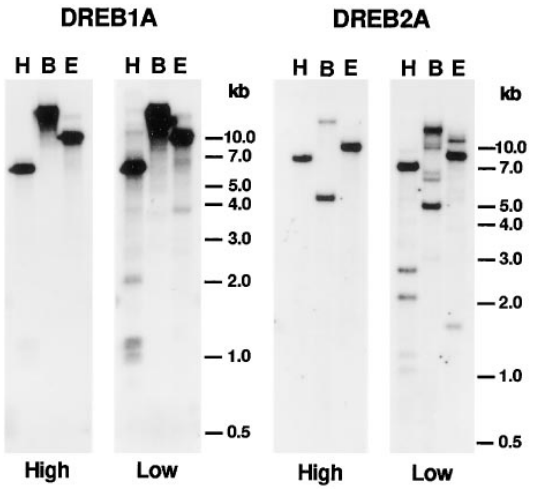

Figure 7. DNA Gel Blot Analysis of Genomic Sequences That Correspond to DREB1A and DREB2A cDNAs.

Genomic DNA was digested with HindIII (H), BamHI (B), and EcoRI (E). A full-length DREB1A or DREB2A cDNA was used as a probe. Filters were washed in either 0.5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% SDS at 50°C (low stringency; Low) or 0.1 × SSC and 0.1% SDS at 65°C (high stringency; High). Numbers at right are molecular length markers in kilobases.

DREB2A proteins were demonstrated to function as transcriptional activators for DRE-dependent transcription not only in yeast cells (Figure 2) but also in transactivation experiments using Arabidopsis leaf protoplasts (Figure 9). These results strongly suggest that both the DREB1A and DREB2A proteins are involved in DRE-dependent expression of the *rd29A* gene. However, they have no significant sequence identity, except for the conserved DNA binding domain, which suggests that they can be assigned to different classes. Two DREB1A-related cDNAs, DREB1B and DREB1C, and one DREB2A-related cDNA, DREB2B, were isolated by screening cDNA libraries prepared from dehydrated, cold-treated, and untreated Arabidopsis plants (Figure 8). These observations suggest that at least five distinct DRE binding proteins in two groups, DREB1 and DREB2, bind to the same target sequence, DRE, and are involved in the activation of the *rd29A* gene in response to dehydration, high-salt, and low-temperature stress.

The bacterially expressed DREB1A and DREB2A proteins specifically bound to the DRE sequence (Figure 5). Both the DREB1A and DREB2A proteins bound to the 71-bp DNA fragment with the DRE sequence but not to the 71-bp DNA fragments with base substitutions in the DRE sequence (M1, M2, and M3). In contrast, the fusion proteins bound to the 71-bp DNA fragments with base substitutions (M4 and M5) in the flanking sequence (Figure 5). These results indicate that the DRE sequence is the target sequence for DNA binding of the DREB1A and DREB2A proteins.

We have shown, using transgenic tobacco and Arabidopsis plants, that the DRE sequence is essential for the transcription of *rd29A* under conditions of drought, high salt, and low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994). We used the same set of 71-bp fragments for the analyses of *cis*-acting elements. The 71-bp fragments with base substitutions (M1, M2, and M3) in the DRE sequence did not function in dehydration-induced expression, whereas the 71-bp fragments with base substitutions (M4 and M5) in the flanking sequence responded to dehydration stress in transgenic plants. These results coincide with the DNA

A

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DREB1A  MNSFSAFSEMFGSDYESSVSSGGDYIPTLASSCPKKPAGR   40
DREB1B  ****************-P--Q****C*T**T*********   37
DREB1C  ****************SPVSS****S*K**T*********   40

DREB1A  KKFRETRHPIYRGVRRRNSGKWVCEVREPNKKTRIWLGTF   80
DREB1B  ***************Q*******S*V**************   77
DREB1C  ***************Q*******C*L**************   80

DREB1A  QTAEMAARAHDVAALALRGRSACLNFADSAWRLRIPESTC  120
DREB1B  **************L************************  117
DREB1C  **************I************************  120

DREB1A  AKDIQKAAAEAALAFQDEMCDATT-DHGFDMEETLVEAIY  159
DREB1B  **D**********A****T*DT**TN**L*****M*****  157
DREB1C  **E**********N****M*HM**DA**L*****L*****  160

DREB1A  TAEQSENAFYMHDEAMFEMPSLLANMAEGMLLPLPSVQWN  199
DREB1B  *P***EG****DE*T*FG*PT**D********P******  197
DREB1C  *P***QD****DE*A*LG*SS**D*********S******  201

DREB1A  HNHEVDGDDDDVSLWSY                        216
DREB1B  H*YDGE*-*G*******                        213
DREB1C  Y*FDVE*-*D*******                        217
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B

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DREB2A  MAVYDQSGDRNRTQIDTSRKRKSRSRGDGTTVAERLKRWK   40
DREB2B  ****E*T*----*E-Q-PK*****A*AG*L***D***K**   34

DREB2A  EYNETVE--EV--STK-KRKVPAKGSKKGCMKGKGGPENS   75
DREB2B  ****I**ASA*KEGE*P*******************D**   74

DREB2A  RCSFRGVRQRIWGKWVAEIREPNRGSRLWLGTFPTAQEAA  115
DREB2B  H*********************KI*T**********EK**  114

DREB2A  SAYDEAAKAMYGPLARLNFPRSDASEVTSTSSQSEVCTVE  155
DREB2B  *******T****S*******Q*VG**F*************  154

DREB2A  -TP-GC--VHVKTEDPDCESKPFSG--GVEPMYCLENGAE  189
DREB2B  NKAVV*GD*C**H**T****N***QILD*REES*GTRPDS  194

DREB2A  -EM-KRGVKAD-K-HWLSEFEHNYWSDILKEKEKQKEQGI  225
DREB2B  CTVGHQDMNSSLNYDL*L***QQ**GQV*Q****P*QEE-  233

DREB2A  VETCQQQQQDSLSVADYGWPNDVDQSHLDSSDMFDVDELL  265
DREB2B  -*EI*****EQ-QQQQLQ-*DLLTVADYGWPWSN*IVNDQ  270

DREB2A  RDLNGDDVFAGLNQDRYPGNSVANGSYRPESQQSGFDPLQ  305
DREB2B  TSWDPNEC*-DI*-E-LL*D-LNEPG-PHQ**D-Q-NHVN  303

DREB2A  SLNYGIPPFQLEGKDGNGFFDDLSYLDLEN            335
DREB2B  *GS*DLH*LH**PH**HE-*NG**S**I--            330
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Figure 8. Comparison of the Deduced Amino Acid Sequences of the DREB1 and DREB2 Families.

Asterisks represent identical amino acid residues, and dashes indicate gaps introduced to maximize alignment. The underlined regions indicate the EREBP/AP2 DNA binding domains. A conserved Ser/Thr–rich region in DREB2A and DREB2B is indicated by a dashed underline.
(A) Comparison of DREB1A, DREB1B, and DREB1C.
(B) Comparison of DREB2A and DREB2B.

binding specificity of the two DREB proteins to the DRE sequence (Figure 5).

Both the DREB1A and DREB2A proteins contain a typical EREBP/AP2 DNA binding motif (Figure 4), which is found in tobacco EREBPs (Ohme-Takagi and Shinshi, 1995) and Arabidopsis AP2 (Jofuku et al., 1994). Recently, the EREBP/AP2 DNA binding motif also has been found in various plant regulatory genes, such as Arabidopsis *TINY* (Wilson et al., 1996), *CBF1* (Stockinger et al., 1997), *AtEBP* (Buttner and Singh, 1997), and *AINTEGUMENTA* (Elliott et al., 1996; Klucher et al., 1996), maize *Glossy15* (Moose and Sisco, 1996), and tomato *Pti*s (Zhou et al., 1997).

These genes are divided into two classes based on the number of the EREBP/AP2 motifs. One class includes *AP2*, *AINTEGUMENTA*, and *Glossy15*, each of which encodes a protein containing two EREBP/AP2 motifs. The other class includes *EREBP*s, *TINY*, *CBF1*, *Pti*s, *AtEBP*, and *DREB*s, each of which encodes a protein with only one EREBP/AP2 motif. The *EREBP*s, *Pti*s, and *AtEBP* in the second class specifically bind to the GCC box sequence containing the core GCCGCC sequence, which is present in the promoter region of a large number of ethylene-inducible genes encoding pathogenesis-related proteins (Ohme-Takagi and Shinshi, 1995). The DREB1A, DREB2A, and CBF1 proteins specifically bind to the DRE/C repeat sequence containing the core sequence, PuCCGAC. These sequences resemble the GCC box and contain CCGNC as a common core sequence.

We compared the amino acid sequences of the DNA binding domains of the DREB1A, DREB2A, and CBF1 proteins and found consensus amino acids in their DNA binding domains (Figure 4, Consensus). All of the consensus amino acids are conserved in the DNA binding domains of *EREBP*s, *Pti*s, and *AtEBP,* except for the fourteenth valine (V) and nineteenth glutamate (E) in the binding domain of DREB1A and DREB2A (Figure 4). These two amino acids are also conserved in the DREB1A and DREB2A homologs (Figure 8). These conserved amino acids in the binding domains of DREB1A, DREB2A, and their homologs, including CBF1, may be important for binding specificity to the target sequence, PuCCGAC. The EREBP/AP2 motif contains an 18–amino acid core region that has been proposed to form an amphipathic α-helix; the latter could play a role in protein–protein interactions to facilitate DNA binding (Okamuro et al., 1997). The region of DREB1A and DREB2A proteins also theoretically is capable of forming the amphipathic α-helix structure.

Expression of *DREB1A*, *DREB1B* (*CBF1*), and *DREB1C* was strongly induced by low-temperature stress, whereas that of *DREB2A* and *DREB2B* was induced by dehydration and high-salt stress (Figure 6; Z.K. Shinwari, K. Nakashima, S. Miura, M. Kasuga, K. Yamaguchi-Shinozaki, and K. Shinozaki, unpublished data). The *CBF1* gene is identical to the *DREB1B* gene. We demonstrated that *DREB1B*/*CBF1* as well as *DREB1A* and *DREB1C* gene expression is induced by low temperature, whereas Stockinger et al. (1997) showed that the *CBF1* gene is constitutively expressed even under unstressed conditions. This difference may be due to differences in stress treatment or growth conditions of Arabidopsis plants. *rd29A* gene expression was induced by dehydration, high-salt, and low-temperature stress (Figure 6). These results suggest that the DREB1A-related proteins function in the DRE/C repeat–dependent expression of *rd29A* during low-temperature stress, whereas the DREB2A-related proteins are involved in the expression of *rd29A* during dehydration and high-salt stress. The expression of the *DREB1A* genes during low-temperature stress precedes that of the *rd29A* gene (Figure 6). Furthermore, the induction

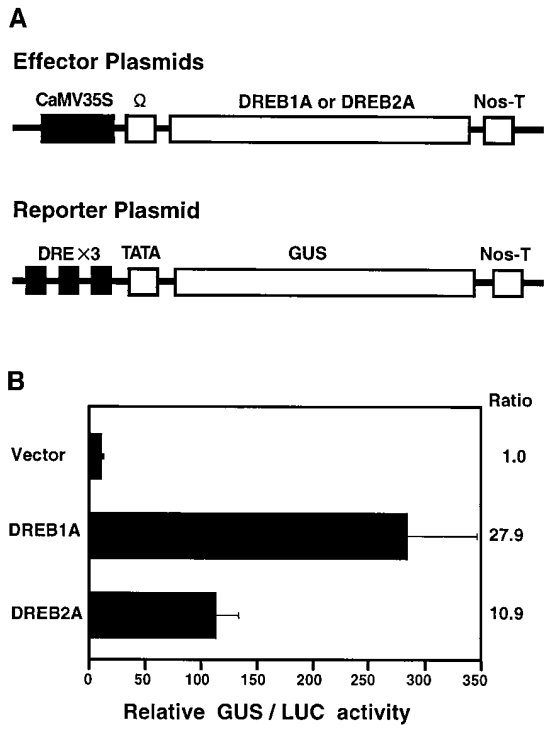

Figure 9. Transactivation of the *rd29A* Promoter–*GUS* Fusion Gene by DREB1A and DREB2A Proteins by Using Arabidopsis Protoplasts.

(A) Schematic diagram of the effector and reporter constructs used in cotransfection experiments. The effector constructs contain the CaMV 35S promoter and TMV Ω sequence (Gallie et al., 1987) fused to the DREB1A or DREB2A cDNA. Nos-T indicates the polyadenylation signal of the gene for nopaline synthetase. The reporter construct contains the 71-bp fragments of the *rd29A* promoter tandemly repeated three times (DRE × 3). The promoter was fused to the −61 *rd29A* minimal TATA promoter–*GUS* construct.

(B) Transactivation of the *rd29A* promoter–*GUS* fusion gene by the DREB1A and DREB2A proteins. The reporter gene was transfected with each effector plasmid or the vector as control treatments. To normalize for transfection efficiency, the CaMV 35S promoter–luciferase (LUC) plasmid was cotransfected in each experiment. Bars indicate the standard error of three replicates. Ratios indicate the multiplicities of expression compared with the value obtained with the pBI35SΩ vector.

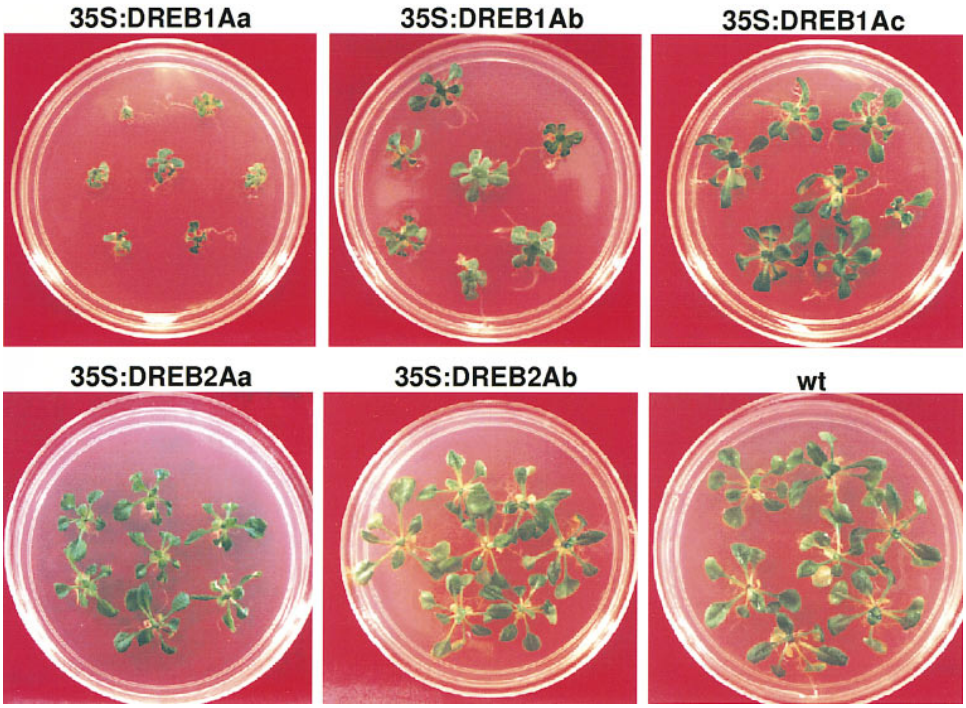

Figure 10. Effects of Overexpressing DREB1A and DREB2A cDNAs in Transgenic Plants.

Shown are 3-week-old seedlings carrying the 35S:DREB1A transgene with a variety of dwarf phenotypes (35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac), those carrying the 35S:DREB2A transgene with growth retardation (35S:DREB2Aa and 35S:DREB2Ab), and 3-week-old seedlings carrying pBI121 (wt).

of *rd29A* expression by high-salt and low-temperature stress was inhibited by half when cycloheximide was used (data not shown), whereas the induction of *rd29A* expression by exogenous ABA treatment was not inhibited (Yamaguchi-Shinozaki and Shinozaki, 1993). These results suggest that induction of the DREB proteins by these stresses is required for the expression of *rd29A*.

However, expression of the *rd29A* gene was induced rapidly by high salt (within 10 min) and dehydration (within 20 min), suggesting that the DREB2A-related transcription factors may be activated directly by these stresses. Indeed, we detected *DREB2A* mRNA in the unstressed control plants. However, we could not detect *DREB1A* mRNA in the unstressed plants (Figure 6B). These observations indicate that both induction and modification of the DREB2A transcription factors are needed for the transactivation of the *rd29A* gene under dehydration and high-salt stress conditions and that the induction of DREB1A transcription factors is important for the expression of *rd29A* under low-temperature stress conditions (Figure 13).

The above-mentioned hypothesis is supported by our analyses of transgenic plants that overexpressed the DREB1A or DREB2A cDNAs. The *rd29A* mRNA as well as the *DREB1A* mRNA accumulated in 35S:DREB1Aa transgenic plants that overexpressed the DREB1A cDNA and revealed a severe dwarf phenotype under unstressed growth conditions (Figures 10 and 11). The level of accumulated *DREB1A* mRNA correlated with the level of *rd29A* mRNA and the phenotypic changes of growth retardation of the transgenic plants (35S:DREB1Ab and 35S:DREB1Ac; Figures 10 and 11). The expression of the *rd17*/*cor47* gene, which is induced by dehydration and low temperature (Gilmour et al., 1992; Iwasaki et al., 1997), was also observed in the 35S:DREB1A plants under unstressed conditions (data not shown). The overproduction of the DREB1A-related proteins is enough to induce the expression of target genes. TINY has sequence similarity with DREBs and CBF1 (Figure 4). Ectopic overexpression of the TINY protein by the 35S promoter resulted in a semidominant dwarf phenotype (Wilson et al., 1996). This phenotype may be due to an effect similar to that causing overexpression of DREB1A in transgenic plants. The 35S:DREB1A transgenic plants revealed freezing and dehydration tolerance (Figure 12) as well as growth retardation. This may have been due to the overexpression of stress-inducible genes that are controlled by the DREB proteins under unstressed conditions. Overproduction of stress-related proteins is likely to make these transgenic plants more stress tolerant under normal growth

conditions, which may cause growth retardation of the plants. Independently, Jaglo-Ottosen et al. (1998) reported that CBF1 overexpression also enhances freezing tolerance.

In contrast, 35S:DREB2A transgenic plants that overexpressed the DREB2A cDNA revealed weak phenotypic changes in growth retardation experiments (35S:DREB2Aa; Figure 10). In 35S:DREB2A transgenic plants, *rd29A* mRNA did not accumulate significantly, although *DREB2A* mRNA accumulated even under unstressed conditions (Figure 11). Expression of the DREB2A protein is not sufficient for the induction of the *rd29A* gene. Modification, such as phosphorylation of the DREB2A protein, seems to be necessary for it to function in response to dehydration. The DREB2A and DREB2B proteins contain a conserved Ser/Thr–rich region adjacent to the EREBP/AP2 DNA binding domain, although the DREB1A-related proteins do not (Figure 8). We are investigating whether this Ser/Thr–rich region is phosphorylated under dehydration conditions.

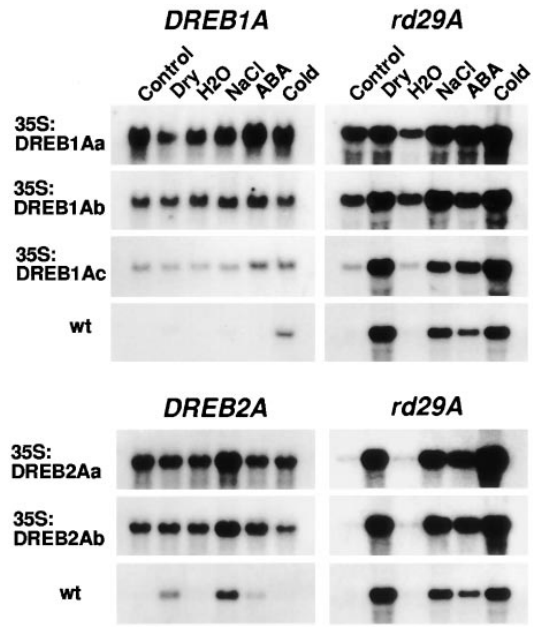

Figure 11. Expression Analyses of the *DREB1A*, *DREB2A*, and *rd29A* Genes in Transgenic Plants.

RNA gel blotting was conducted to measure the amount of *DREB1A*, *DREB2A*, or *rd29A* mRNA in transgenic Arabidopsis plants carrying the 35S:DREB1A transgene (35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac), those carrying the 35S:DREB2A transgene (35S:DREB2Aa and 35S:DREB2Ab), and those carrying pBI121 (wt). Transgenic plants were dehydrated (Dry), transferred from agar plates for hydroponic growth in water (H_2O), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), transferred from agar plates for hydroponic growth in 100 μM ABA (ABA), transferred to 4°C (Cold), and then treated for 5 hr under each condition or were untreated (Control). DNA fragments for the DREB1A and DREB2A cDNAs or the 3′ flanking region of *rd29A* were used as probes.

Figure 13 summarizes a model of the role of the two DREB proteins in the separation of two different signaling pathways under drought and cold stress conditions. Expression of DREB1A and its homologs is induced by low temperature, and the accumulated DREB1A homologs in turn transactivate the DRE-dependent gene expression of *rd29A.* Therefore, the expression of the *rd29A* gene is slower than that of DREB1A under low-temperature conditions. Overexpression of DREB1A in transgenic Arabidopsis activates the expression of the *rd29A* gene under normal unstressed conditions. Transgenic Arabidopsis plants overproducing DREB1A revealed abnormal stressed phenotypes. These observations indicate that the transcriptional activation of the *rd29A* gene is controlled directly by the induction of the DREB1A protein in Arabidopsis plants. In contrast, expression of DREB2A and its homolog is induced by drought and high salt; however, these genes are also expressed in unstressed control plants at low levels. The induced DREB2A and its homolog then transactivate DRE-dependent gene expression under drought and high-salt conditions.

The expression of the *rd29A* gene as well as the *DREB2A* gene is induced rapidly by drought and high-salt stress. In this case, the induction of DREB2A proteins alone is not sufficient for the induction of *rd29A* gene expression; the DREB2A protein probably requires modification (such as phosphorylation) for its function because overexpression of DREB2A had little effect on the expression of the *rd29A* gene under unstressed conditions (Figure 11). A stress signal is necessary for the modification of the DREB2A homologs to their active forms in the transcription of the *rd29A* gene under water-deficient conditions. Both the DREB1A and DREB2A families of proteins bind to the same *cis*-acting element, DRE, and activate the gene expression, but these two families of proteins function in different signal transduction pathways under low-temperature and dehydration stress.

METHODS

Plant Materials and Stress Treatments

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 3 weeks, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). Dehydration, high-salt, and cold stress treatments and treatment with abscisic acid (ABA) were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). The plants were subjected to the stress treatments for various periods and then frozen in liquid nitrogen for further analyses.

Construction of Reporter Plasmids for Yeast One-Hybrid Screening

The 71-bp polymerase chain reaction fragment between positions −215 and −145 in the *rd29A* promoter, which contains a dehydration-responsive element (DRE) and HindIII sites at its 5′ and 3′ ends

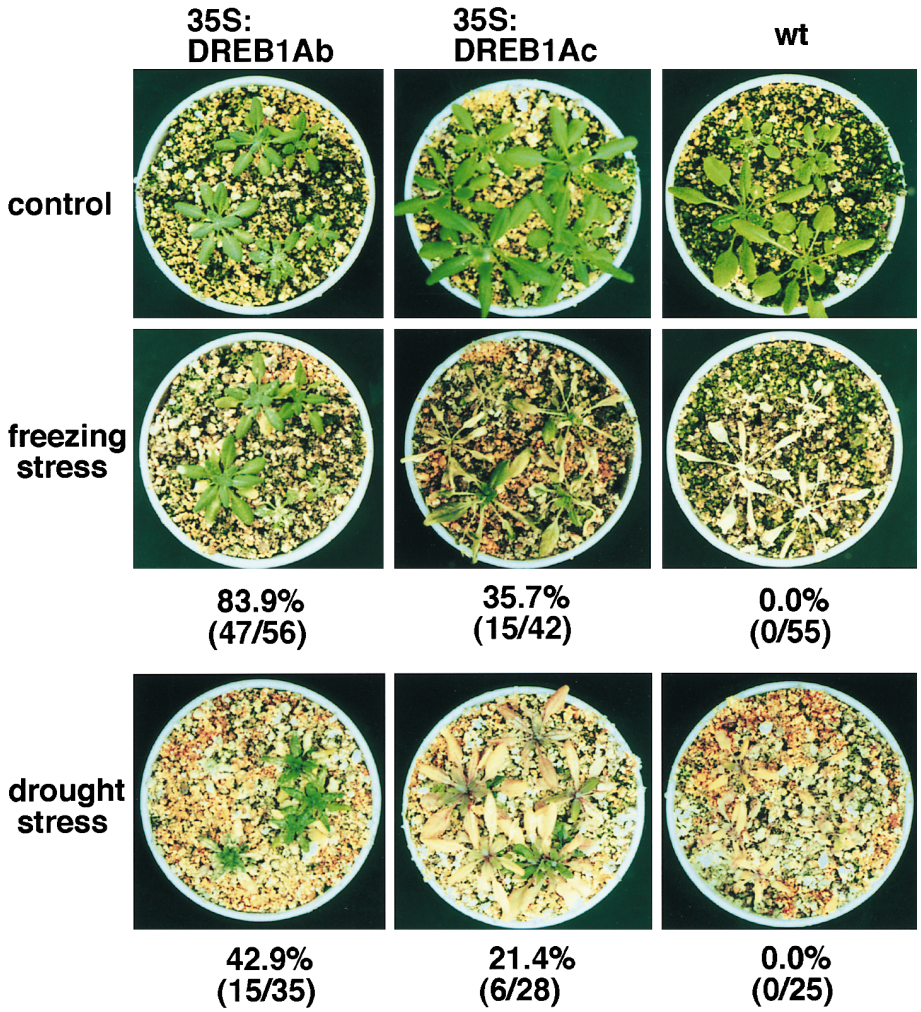

Figure 12. Freezing and Drought Tolerance of the 35S:DREB1Ab and 35S:DREB1Ac Transgenic Plants.

Control, 3-week-old plants growing under normal conditions; freezing stress, plants exposed to a temperature of −6°C for 2 days and returned to 22°C for 5 days; drought stress, water withheld from plants for 2 weeks. Percentages of surviving plants and numbers of surviving plants per total number of tested plants are indicated under the photographs. wt, wild type.

(Yamaguchi-Shinozaki and Shinozaki, 1994), was ligated into four tandemly repeated copies and then inserted into the HindIII site in the multicloning site (MCS) of the pBluescript II SK− (Stratagene, La Jolla, CA) vector. The fragment containing four tandem copies of the 71 bp was excised by EcoRI and HincII from the pBluescript II SK− vector and cloned into MCS upstream from the *HIS3* minimal promoter in the pHISi-1 expression vector, which had been digested with EcoRI and MluI (Clontech, Palo Alto, CA). The same fragment was excised by EcoRI and SalI from the pBluescript II SK− vector and cloned into MCS upstream from the *lacZ* minimal promoter in the pLacZi expression vector (Clontech), which had been digested with the same enzymes. Two kinds of expression plasmids were transformed simultaneously into yeast YM4271 strain (Figure 1). Yeast transformants containing the *HIS3* and *lacZ* reporter genes were obtained in selective medium plates (without His and Ura). The yeast transformant strains that could not grow under 10 mM 3-aminotriazole (3-AT) were used to screen the cDNA libraries.

Construction of Activation Domain–Tagged cDNA Libraries Derived from Dehydrated and Undehydrated Arabidopsis Rosette Plants

Twenty grams of whole rosette plants grown on GM agar plates for 3 weeks was used to prepare cDNA libraries. In the preparation of a cDNA library from dehydrated plants, we dehydrated harvested Arabidopsis plants at room temperature and 60% humidity under dim light for 2 hr and then froze them in liquid nitrogen. The weight of the plants decreased 22% after 2 hr of dehydration. In the preparation of a cDNA

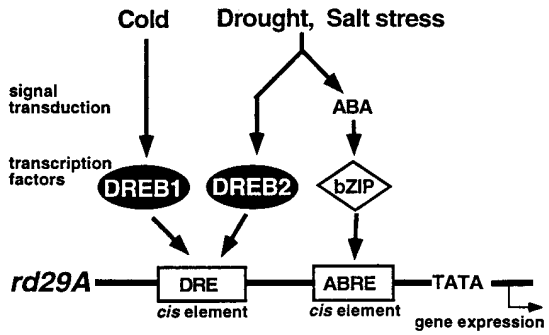

Figure 13. A Model for the Induction of *rd29A* Gene Expression under Dehydration, High-Salt, and Low-Temperature Conditions.

There are at least two independent signal transduction pathways—ABA independent and ABA responsive—between environmental stress and expression of the *rd29A* gene. The DRE functions in the ABA-independent pathway, and the ABA-responsive element (ABRE) is one of the *cis*-acting elements in the ABA-responsive induction of *rd29A.* Two independent families of DRE binding proteins, DREB1 and DREB2, function as *trans*-acting factors and separate two signal transduction pathways in response to cold, and drought and high-salinity stresses, respectively. bZIP, basic leucine zipper.

library from cold-treated plants, we transferred Arabidopsis plants to 4°C for 24 hr and harvested them. Total RNA, poly(A)$^+$ RNA, and cDNAs were prepared as described previously (Yamaguchi-Shinozaki et al., 1992). The cDNAs were ligated with the EcoRI-NotI-BamHI adapter fragment (Amersham) and then cloned into the EcoRI site in MCS downstream of the GAL4 activation domain in the pAD-GAL4 phagemid vector containing the *LEU2* reporter gene (Stratagene).

Yeast One-Hybrid Screening of Arabidopsis cDNA Libraries

Approximately 0.8×10^6, 1.2×10^6, and 1.5×10^6 yeast transformants were screened using cDNA libraries prepared from dehydrated, cold-treated, and unstressed Arabidopsis plants, respectively, according to the manufacturer's protocol (Clontech Matchmaker one-hybrid system). We obtained 203 positive colonies from selective medium plates (without His, Ura, and Leu but containing 10 mM 3-AT). Growth of these clones was examined at 30, 45, and 60 mM 3-AT. The β-galactosidase activities of the clones were then further analyzed. Finally, 41 clones, which grew normally on the 60 mM 3-AT plate and had β-galactosidase activity, were selected from the 203 positive clones. The cDNA of these 41 clones was isolated with a yeast DNA isolation system (Stratagene). The cDNA inserts were excised with EcoRI from the pAD-GAL4 plasmids and ligated into the pBluescript II SK− vector for sequencing.

Construction of Reporter Plasmids for the Transactivation Experiment with Yeast

To analyze transactivation activity of isolated cDNA clones, we fused three tandemly repeated copies of the wild-type or the mutated 71-bp fragment containing the DRE sequence to the MCS upstream from the *HIS3* minimal promoter in the pHISi-1 expression vector and the *lacZ* minimal promoter in the pLacZi expression vector, as shown in Figure 2. The DRE sequence TACCGACAT in the mutated 71-bp fragment was replaced with TATTTTCAT (Figure 5A, M2; Yamaguchi-Shinozaki and Shinozaki, 1994). These plasmids were transformed into the yeast YM4271 strain and used for the transactivation experiment with yeast (Figure 2).

Preparation of Glutathione *S*-Transferase Fusion Proteins and Gel Mobility Shift Assays

A 429-bp (119 to 547) fragment of the DREB1A cDNA and a 500-bp (167 to 666) fragment of the DREB2A cDNA were prepared by polymerase chain reaction and cloned into the EcoRI-SalI sites of the pGEX-4T-1 vector. The primer sets used for the amplification of the DREB1A and DREB2A cDNA fragments were 5′-CAGAGAATTCCGGATCCCAATGAACTCATTTTCTGCT-3′ and 5′-CCGCACTCGAGGTCGACCGTCGCATCACACATCTC-3′ and 5′-GATCCGAATTCATGGCAGTTTATGATCAGAGTGG-3′ and 5′-CAGCACTCGAGGTCGACGGATCCTCTGTTTTCAC-3′, respectively. The recombinant pGEX-4T-1 plasmids (Pharmacia) were transformed to *Escherichia coli* Blue XL-1. Production and purification of the glutathione *S*-transferase (GST) fusion proteins were performed as described previously (Urao et al., 1993). The 71-bp fragments containing DRE of the *rd29A* promoter with or without base substitutions were labeled with a ^{32}P-dCTP, as described previously. Gel mobility shift assays were conducted as described previously (Urao et al., 1993).

DNA and RNA Gel Blot Analyses

DNA gel blot hybridization and RNA gel blot hybridization were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Transactivation Experiments with Protoplasts

Effector plasmids used in the transient transactivation experiment were constructed with DNA fragments containing the *DREB1A* or *DREB2A* coding regions that were cloned into polylinker sites of the plant expression vector pBI35SΩ, which was derived from pBI221 (Clontech). The pBI35SΩ vector was constructed as described previously (Abe et al., 1997). To construct 35S–Ω–DREB1A and 35S–Ω–DREB2A, we cloned the NotI fragment containing the coding region of DREB1A or DREB2A cDNA into the NotI site of the pBI35SΩ vector. To construct a reporter plasmid, we replaced the 35S promoter of pBI221 (Yamaguchi-Shinozaki and Shinozaki, 1994) with the *rd29A* minimal TATA promoter, and we then ligated the 71-bp fragment of the *rd29A* promoter into the HindIII site located upstream from the *rd29A* minimal TATA promoter with three tandem copies.

Isolation of Arabidopsis mesophyll protoplasts and polyethylene glycol–mediated DNA transfection were performed as described previously (Abel and Theologis, 1994). β-Glucuronidase (GUS) activity was measured as picomoles of product formed per minute per milligram of protein by using the standard protocol (Jefferson et al., 1986). Luciferase assays were performed using a PicaGene luciferase assay kit (Toyo-Ink, Tokyo, Japan), according to the manufacturer's instructions. Protein concentration was determined by the Bradford method (Bio-Rad).

Transgenic Plants Overexpressing the DREB cDNAs

Plasmids used in transformation of Arabidopsis were constructed with DREB1A or DREB2A full-length cDNA that was cloned into a polylinker site of a binary vector, pBI2113Not, which was derived from pBI2113 (Mituhara et al., 1996) in the sense orientation. For the construction of the pBI2113Not vector, pBI2113 was digested with SmaI and SacI to delete the GUS coding region and ligated with a SmaI–NotI–SacI polylinker (Takara, Tokyo, Japan). To construct the 35S:DREB1A and 35S:DREB2A plasmids, the EcoRV-SmaI fragment of the DREB1A cDNA and the SacI-EcoRV fragment of the DREB2A cDNA were cloned into the SacI-SmaI or SmaI site of the pBI2113Not vector, respectively. The constructs were introduced into *Agrobacterium tumefaciens* C58, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Arabidopsis plants used for transformation were grown in 8-cm pots filled with soil under continuous illumination at ~2500 lux at 22°C for 6 weeks. Plants were transformed by the vacuum infiltration method, as described by Bechtold et al. (1993).

Freezing and Drought Stress Tolerance of Transgenic Plants

Plants were grown in 9-cm pots filled with a 1:1 mixture of perlite/vermiculite. They were grown under continuous illumination of ~2500 lux at 22°C. Three-week-old plants were exposed to freezing and drought stress. Freezing stress was conducted by exposure of plants to a temperature of −6°C for 2 days and returned to 22°C for 5 days. Drought stress was conducted by withholding water for 2 weeks.

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